

### REMARKS/ARGUMENTS

With this amendment, claims 1-3, 5, 7-11, and 14 are pending. Claims 4, 6, 12-13, and 15-30 are cancelled without prejudice. For convenience, the Examiner's rejections are addressed in the order presented in a September 23, 2008, Office Action.

#### **I. Sequence compliance**

The Office Action asserts that a variety of sequence are recited in the specification without identification in the sequence listing. In particular, the Office Action objects to sequences in the figures that allegedly are not identified in the sequence listing.

The requirement for SEQ ID NO's for sequences disclosed in figures is addressed in MPEP 2422.02.

[W]hen a sequence is presented in a drawing, regardless of the format or the manner of presentation of that sequence in the drawing, the sequence must still be included in the Sequence Listing and the sequence identifier ("SEQ ID NO:X") must be used, **either** in the drawing **or** in the Brief Description of the Drawings. *Emphasis added.*

Thus, the SEQ ID NO: for a sequence found in a drawing can be provided in the drawing or in a description of the drawing.

Applicants direct the Examiner's attention to substitute sheets filed with the application. For the Examiner's convenience, Applicants have downloaded copies of the substitute sheets from PAIR, both a final version and a marked up copy, and include them with this response as Exhibit A. The figure legends for Figures 6-14, 24A-B, 30, 35, 38A-C, and 41-44 are found in the Brief Description of the Drawings. See, *e.g.*, paragraphs [62]-[70], [80], [82], [91], [94], and [97]-[100] of the substitute sheets. Each figure legend includes sequence identifier(s) for the sequence(s) found in the described figure. Paragraph [0332] of the substitute sheets also includes SEQ ID NOs. Therefore, Applicants respectfully assert that the application as filed is in compliance with the sequence rules.

### III. Rejections under 35 U.S.C. §103(a)

Claims 1-3, 5, 7-11, and 14 are rejected over various combinations of references. To establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference must teach or suggest all the claims limitations. MPEP§2143. Recently, in reviewing this standard, the Supreme Court noted that any analysis supporting a rejection under § 103(a) must be made explicit, and that it is "important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the [prior art] elements in the manner claimed." *KSR Intl Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1396 (U.S. 2007). "This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known." *Id.*

While the Court warned against a "rigid application" of the TSM test, the Court also found that these questions could provide a "helpful insight" in determining whether the claimed subject matter is obvious under § 103(a). *Id.* at 1396-1397. *See also*, Memorandum to Technology Directors from Margaret A. Focarino, Deputy Commissioner for Patent Operations, May 3, 2007. To the extent the rejection applies to the amended claims, Applicants respectfully traverse the rejection.

Claims 1-2, 7, 10-11, and 14 are rejected as obvious over Paulson *et al.* (US Patent No. 5,858,751) in view of Hellman *et al.*, *Protein Expr. Pur.* 6:56-62 (1995) and Clark, *Curr. Opin. Biotech.* 12:202-207 (2001). According to the Office Action, Paulson *et al.* teaches methods of expression of eukaryotic  $\alpha(2,3)$ sialyltransferase (ST3Gal3) proteins; Clark teaches methods of isolating and refolding insoluble protein from inclusion bodies; and Hellman *et al.* teaches addition of MBP domains to proteins. The Office Action alleges that the cited references "define the status of the prior art in the successful method of expression, isolation, solubilization

and refolding of sialyltransferase from inclusion body of prokaryotes expression system.” Office Action at page 6.

The Office Action overstates the predictability of obtaining an active enzyme after refolding from an inclusion body. The prokaryotic expression and refolding of eukaryotic ST3Gal3 from an inactive enzyme in inclusion bodies to a soluble active enzyme would be considered a surprising result to those of skill. None of the cited references disclose or suggest that an soluble, active ST3Gal3 enzyme could be obtained by refolding an insoluble enzyme from an inclusion body after expression in a prokaryote. Moreover, the specification demonstrates that refolding results are not predictable. *See, e.g.*, Table 2 at page 73, which demonstrates that GSH/GSSG is a surprisingly effective refolding agent. Thus, at a minimum, claim 10, which recites GSH/GSSG, overcomes this rejection.

Claim 3 is rejected as obvious over Paulson *et al.*, in view of Hellman *et al.*, and Clark, in further view of Ramakrishnan *et al. J. Biol. Chem.* 276:37665-37671 (2001). Paulson *et al.*, Hellman *et al.*, and Clark are discussed above. According to the Office Action, Ramakrishnan *et al.* teaches mutation of an unpaired cysteine in a  $\beta$ -galactosidase enzyme increased the yield of refolded enzyme. As argued above, none of the cited references, including Ramakrishnan *et al.*, disclose or suggest that an soluble, active ST3Gal3 enzyme could be obtained by refolding an insoluble enzyme from an inclusion body after expression in a prokaryote.

Claim 5 is rejected as obvious over Paulson *et al.*, in view of Hellman *et al.*, and Clark, in further view of Nilsson *et al. Protein Expr. Pur.* 11:1-16 (1997). Paulson *et al.*, Hellman *et al.*, and Clark are discussed above. According to the Office Action, Nilsson *et al.* teaches purification of a fusion protein using an affinity tag and an appropriate column. As argued above, none of the cited references, including Nilsson *et al.*, disclose or suggest that an soluble, active ST3Gal3 enzyme could be obtained by refolding an insoluble enzyme from an inclusion body after expression in a prokaryote.

Claims 8 and 9 are rejected as obvious over Paulson *et al.*, in view of Hellman *et al.*, and Clark. Paulson *et al.*, Hellman *et al.*, and Clark are discussed above. According to the Office Action, use of a multienzyme system was obvious to those of skill in the art. The Office Action overstates the predictability of simultaneously refolding more than one enzyme to arrive at a reaction mixture with multiple correctly refolded and active enzymes. The prokaryotic expression and simultaneous refolding of multiple eukaryotic glycosyltransferases from inactive enzymes in inclusion bodies to soluble active enzymes would be considered a surprising result to those of skill. Moreover, the specification demonstrates that refolding results are not predictable. For example, Figure 5 compares GlycoPEGylation of EPO using either GalT1, GnT1, and ST3GalIII, each separately expressed in a eukaryotic system (lane 2), or GalT1, GnT1, and St3GalIII, each separately expressed in inclusion bodies in *E. coli* and then solubilized, combined and refolded to active enzymes (lane 5). The enzymes expressed in eukaryotic systems, were expressed under conditions that encourage proper refolding, and were properly refolded and active before being mixed. In contrast, the bacterially expressed glycosyltransferases were not refolded before being combined, decreasing the chances of proper refolding. Therefore, those of skill would consider the activity of refolded, eukaryotic glycosyltransferases expressed in bacterial cells to be a surprising result.

In view of the above amendments and remarks, withdrawal of the rejection for alleged obviousness is respectfully requested.

Appl. No. 10/587,769  
Amdt. dated December 22, 2008  
Reply to Office Action of September 23, 2008

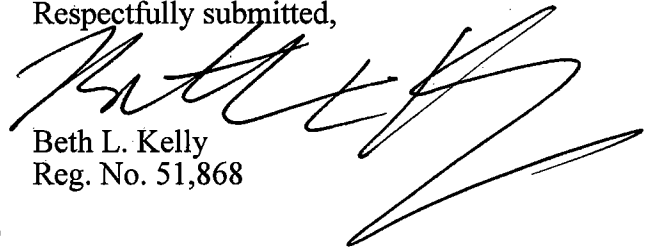
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**CONCLUSION**

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,



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# **EXHIBIT A**

[0061] Figure 5 provides the results of an assay of GlycoPEGylation of EPO using the refolded SuperGlycoMix. Lanes are as follows: (1) MW markers, SeeBlue2 Invitrogen,(250, 148, 98, 64, 50, 36, 22, 16, 6 kD); (2) Positive control with EPO, + NSO expressed GalT1, BV GnT1, Aspergillus ST3GalIII and sugar nucleotides; (3) Negative control, Same as 2 without UDP-GlcNAc; (4) EPO, Purified and separately refolded MBP-GalT1( $\Delta$ 129) C342T, Refolded MBP-GnT1( $\Delta$ 103), and *Aspergillus niger* expressed ST3GalIII; (5) EPO, SuperGlycoMix (mixture of MBP-ST3GalIII, MBP-GalT1( $\Delta$ 129) C342T, MBP-GnT1( $\Delta$ 103)C123A and sugar nucleotides.

[0062] Figure 6 provides an alignment of a human GnT1 amino acid sequence (top line, NP\_002397; SEQ ID NO:1) and a rabbit GnT1 amino acid sequence (bottom line, P27115; SEQ ID NO:2). The conserved unpaired cysteines are underlined and in bold text.

[0063] Figure 7 provides the amino acid sequence (SEQ ID NO:3) of a GnT1 Cys121Ser mutant and a nucleic acid sequence (SEQ ID NO:4) that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrsldkllh.... (SEQ ID NO:5), where the bold residue is mutated from the wild-type cysteine.

[0064] Figure 8 provides the amino acid sequence (SEQ ID NO:6) of a GnT1 Cys121Asp mutant and a nucleic acid sequence (SEQ ID NO:7) that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrldkllh... (SEQ ID NO:8), where the bold residue is mutated from the wild-type cysteine.

[0065] Figure 9 provides the amino acid sequence (SEQ ID NO:9) of a GnT1 Cys121Thr mutant and a nucleic acid sequence (SEQ ID NO:10) that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrtldkllh...(SEQ ID NO:11), where the bold residue is mutated from the wild-type cysteine.

[0066] Figure 10 provides the amino acid sequence (SEQ ID NO:12) of a GnT1 Cys121Ala mutant and a nucleic acid sequence (SEQ ID NO:13) that encodes the mutant GnT1 protein.

The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvr**ra**ldkllh... (SEQ ID NO:14), where the bold residue is mutated from the wild-type cysteine.



[0067] Figure 11 provides the amino acid sequence (SEQ ID NO:15) of a GnT1 Arg120Ala, Cys121His mutant and a nucleic acid sequence (SEQ ID NO:16) that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following double mutation: ...stvr**ah**ldkllh... (SEQ ID NO:17), where the bold residue is mutated from the wild-type cysteine.

[0068] Figure 12 provides the amino acid sequence of rat liver ST3GalIII (SEQ ID NO:18). The underlined and italicized sequence was deleted to make the  $\Delta$ 28 deletion.

[0069] Figures 13A and 13B provide full length nucleic acid (SEQ ID NO:20) and amino acid (SEQ ID NO:19) sequences of UDP-N-acetylgalactosaminyltransferase 2 (GalNAcT2). The accession number of the nucleic acid and protein is NM\_004481.

[0070] Figures 14A and 14B provide nucleic acid (SEQ ID NO:22) and amino acid (SEQ ID NO:21) sequences of a  $\Delta$ 51GalNAcT2. The numbering is based on the full length amino acid and nucleic acid sequences shown in Figures 13A and B.

[0071] Figure 15 provides a demonstration of the protein concentration of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Protein concentrations were measured immediately after refolding (light gray bars), after dialysis (dark gray bars), and after concentration (white bars).

[0072] Figure 16 provides a demonstration of the enzymatic activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Activity was measured after dialysis (light gray bars) and after concentration (dark gray bars).

[0073] Figure 17 provides a demonstration of the specific activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Specific activity was measured after dialysis (white bars) and after concentration (dark gray bars).

[0074] Figures 18A and 18B provide results of remodeling of recombinant granulocyte colony stimulating factor (GCSF) using refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. Figure 18A shows the results using a control purified MBP-GalNAcT2(D51), or a negative control that lacked a substrate, or

bacterially expressed MBP-GalNAcT2(D51) that was solubilized at pH 6.5 and refolded at pH 6.5. Figure 18B shows the experimental results.

[0075] Figure 19 provides a profile of refolded MBP-GalNAcT2(D51) proteins after elution from a Q Sepharose XL (QXL) column (Amersham Biosciences, Piscataway, NJ).

5 The top of the figure shows a chromatogram illustrating the elution of MBP-GalNAcT2(D51) from the QXL column. Fraction numbers are indicated on the X-axis and the relative absorbance of each fraction is indicated on the Y-axis. The bottom shows an image of two electrophoretic gels used to visualize the eluted fractions. The contents of each lane on the gel are described in the figure.

10 [0076] Figure 20 provides the GalNAcT2 activity of specific column fractions from the QXL column shown in Figure 19. The most active fractions were applied to a Hydroxyapatite Type I (80µm) (BioRad, Hercules, CA) column.

[0077] Figure 21 provides a profile of refolded MBP-GalNAcT2(D51) proteins after elution from the HA type I column. The top of the figure shows a chromatogram illustrating  
15 the elution of MBP-GalNAcT2(D51) from the HA type I column. Fraction numbers are indicated on the X-axis and the relative absorbance of each fraction is indicated on the Y-axis. The bottom shows an image of an electrophoretic gel used to visualize the eluted fractions. The contents of each lane on the gel are described in the figure.

[0078] Figure 22 provides the GalNAcT2 activity of HA type I eluted fractions.

20 [0079] Figure 23 provides a comparison of purification and activity of ST3Gal3 proteins fused to either an MBP tag or to an MBP-SBD tag.

[0080] Figure 24 provides the amino acid sequences of the MBP-ST3Gal1 fusion protein (SEQ ID NO:23) (A) and the MBP-SBD-ST3Gal1 fusion protein (SEQ ID NO:24) (B).

[0081] Figure 25 provides the sialyltransferase activity of the MBP-ST3Gal3 fusion  
25 protein) and the MBP-SBD-ST3Gal3 fusion protein. positive and negative controls are also shown.

[0082] Figure 26 provides the amino acid sequence of mouse and human ST6GalNAcI proteins fused to MBP. Part A shows the sequence of a mouse truncation fusion: MBP-mST6GalNAcI S127 (SEQ ID NO:25). Part B shows the sequence of a human truncation  
30 fusion: MBP-hST6GalNAcI K36 (SEQ ID NO:26).

- [0083] Figure 27 provides SDS-PAGE gels of O-linked glycosyltransferase enzyme (A) concentrations after co-refolding and the (B) results of an enzyme assay after co-refolding. MBP-GalNAcT2 and MBP-ST3GalI were co-refolded together. Enzyme activity was tested after addition of Core I Gal T1 enzyme. The substrates were IF $\alpha$ -2b and 20K-Peg-CMP-NAN.
- [0084] Figure 28 provides an SDS-PAGE gel showing expression of the native SiaA protein in *E. coli* before and after induction with IPTG.
- [0085] Figure 29 provides an SDS-PAGE gel showing expression of an MBP-SiaA fusion protein in *E. coli* before and after induction with IPTG.
- 10 [0086] Figure 30 provides the amino acid sequence of the full length bovine GalT1 protein (SEQ ID NO:27).
- [0087] Figure 31 depicts GalT1 mutants schematically, as well as a control protein GalT1(40) (S96A+C342T).
- [0088] Figure 32 provides the results of enzymatic assays of the refolded and purified  
15 MBP-GalT1 (D70) protein. The assay measured conversion of LNT2 (Lacto-N-Triose-2) into LNNt (Lacto-N-Neotetraose) using UDP-Gal (Uridine 5'-Diphosphogalactose) as a donor substrate.
- [0089] Figure 33 provides an RNase B remodeling assay of MBP-GalT1 (D70) and a control protein GalT1(40) (S96A+C342T), also referred to as Qasba's GalT1.
- 20 [0090] Figure 34 provides kinetics of glycosylation of RNase B using the refolded and purified MBP-GalT1 (D70) protein or NSO GalT1, a soluble form of the bovine GalT1 protein that was expressed in a mammalian cell system.
- [0091] Figure 35 provides a schematic of the MBP-GnT1 fusion proteins, and depicts the truncations, *e.g.*,  $\Delta$ 103 or  $\Delta$ 35, and the Cys121Ser mutation (top). The bottom of the figure  
25 provides the full length human GnT1 protein (SEQ ID NO:1).
- [0092] Figure 36 provides an SDS-PAGE gel showing in the right panel the refolded MBP-GnT1 fusion proteins: MBP-GnT1(D35) C121A, MBP-GnT1(D103) R120A + C121H, and MBP-GnT1(D103) C121A. The left panel shows GnT1 activities of two different batches (A1 and A2) of refolded MBP-GnT1(D35) C121A at different time points.
- 30 [0093] Figure 37 provides a full length sequence of porcine ST3GalI (SEQ ID NO:28).

[0094] Figure 38 provides full length amino acid sequences for A) human ST6GalNAcTI (SEQ ID NO:29) and for B) chicken ST6GalNAcTI (SEQ ID NO:30), and C) a sequence of the mouse ST6GalNAcTI protein beginning at residue 32 of the native mouse protein (SEQ ID NO:31).

5 [0095] Figure 39 provides a schematic of a number of preferred human ST6GalNAcI truncation mutants.

[0096] Figure 40 shows a schematic of MBP fusion proteins including the human ST6GalNAcI truncation mutants.

10 [0097] Figure 41 provides the full length sequence of human Core 1 GalT1 protein (SEQ ID NO:32).

[0098] Figure 42 provides the sequences of two *Drosophila* Core 1 GalT1 proteins (SEQ ID NOS:33 and 34).

15 [0099] Figure 43 provides the sequences of exemplary bacterial MBP proteins that can be fused to glycosyltransferases to enhance refolding. A. *Yersinia* MBP (SEQ ID NO:35); B. *E. coli* MBP (SEQ ID NO:36); C. *Pyrococcus furiosus* MBP (SEQ ID NO:37); D. *Thermococcus litoralis* MBP (SEQ ID NO:38); E. *Thermatoga maritima* MBP (SEQ ID NO:39); and F. *Vibrio cholerae* MBP (SEQ ID NO:40).

20 [0100] Figure 44 provides an alignment of human GalNAcT1 (SEQ ID NO:41) and GalNAcT2 proteins (SEQ ID NO:19). Because the alignment programs account for sequence insertions or deletions, the numbering of cysteine residues is not the same as mentioned text and published sequences. In the case of hGalNAc-T2 cysteine 227 (published) corresponds to position 235 in the alignment and cysteine 229 (published) is 237 in the alignment. The hGalNAc-T1 cysteines are 212 (published), which corresponds to cysteine 235 (alignment) and 214 (published), which corresponds to cysteine 237 (alignment). The relevant cysteine  
25 residues are indicated by larger font size. Consensus peptides = SEQ ID NOS:42-65.

[0101] Figure 45 shows the position of paired and unpaired cysteine residues in the human ST6GalNAcI protein. Single and double cysteine substitution are also shown, e.g., C280S, C362S, C362T, (C280S + C362S), and (C280S + C362T).

## DEFINITIONS

[0102] The recombinant glycosyltransferase proteins of the invention are useful for transferring a saccharide from a donor substrate to an acceptor substrate. The addition generally takes place at the non-reducing end of an oligosaccharide or carbohydrate moiety  
5 on a biomolecule. Biomolecules as defined here include but are not limited to biologically

[0133] A "fusion protein" refers to a protein comprising amino acid sequences that are in addition to, in place of, less than, and/or different from the amino acid sequences encoding the original or native full-length protein or subsequences thereof.

[0134] Components of fusion proteins include "accessory enzymes" and/or "purification

5 tags." An "accessory enzyme" as referred to herein, is an enzyme that is involved in catalyzing a reaction that, for example, forms a substrate for a glycosyltransferase. An accessory enzyme can, for example, catalyze the formation of a nucleotide sugar that is used as a donor moiety by a glycosyltransferase. An accessory enzyme can also be one that is used in the generation of a nucleotide triphosphate required for formation of a nucleotide sugar, or  
10 in the generation of the sugar which is incorporated into the nucleotide sugar. The recombinant fusion protein of the invention can be constructed and expressed as a fusion protein with a molecular "purification tag" at one end, which facilitates purification of the protein. Such tags can also be used for immobilization of a protein of interest during the glycosylation reaction. Suitable tags include "epitope tags," which are a protein sequence  
15 that is specifically recognized by an antibody. Epitope tags are generally incorporated into fusion proteins to enable the use of a readily available antibody to unambiguously detect or isolate the fusion protein. A "FLAG tag" is a commonly used epitope tag, specifically recognized by a monoclonal anti-FLAG antibody, consisting of the sequence

AspTyrLysAspAspAspLys (SEQ ID NO:66) or a substantially identical variant thereof.

20 Other suitable tags are known to those of skill in the art, and include, for example, an affinity tag such as a hexahistidine peptide (SEQ ID NO:67), which will bind to metal ions such as nickel or cobalt ions. Proteins comprising purification tags can be purified using a binding partner that binds the purification tag, *e.g.*, antibodies to the purification tag, nickel or cobalt ions or resins, and amylose, maltose, or a cyclodextrin. Purification tags also include starch  
25 binding domains, *E. coli* thioredoxin domains (vectors and antibodies commercially available from *e.g.*, Santa Cruz Biotechnology, Inc. and Alpha Diagnostic International, Inc.), and the carboxy-terminal half of the SUMO protein (vectors and antibodies commercially available from *e.g.*, Life Sensors Inc.). Maltose binding domains are preferably used for their ability to enhance refolding of insoluble eukaryotic glycosyltransferases, but can also be used to assist  
30 in purification of a fusion protein. Purification of maltose binding domain proteins is known to those of skill in the art. Starch binding domains are described in WO 99/15636, herein incorporated by reference. Affinity purification of a fusion protein comprising a starch binding domain using

about amino acid residues 32-90. Thus, a truncated human Core1 GalT1 protein can have deletions at the amino terminus of about *e.g.*, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 residues.

5. [0210] Deletion mutations can also be made in an ST3Gal1 protein. For example, the human ST3Gal1 protein includes a stem region from about amino acid residues 18-58. Thus, a truncated human ST3Gal1 protein can have deletions at the amino terminus of about *e.g.*, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, or 58 residues. As another example, the  
10 porcine ST3Gal1 protein includes a stem region from about amino acid residues 28-61. Thus, a truncated porcine ST3Gal1 protein can have deletions at the amino terminus of about *e.g.*, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, or 61 residues.

- [0211] Deletion mutations can also be made in a GalNAcT2 protein. For example, the rat  
15 GalNAcT2 protein includes a stem region from about amino acid residues 40-95. Thus, a truncated rat GalNAcT2 protein can have deletions at the amino terminus of about *e.g.*, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 residues.

- 20 [0212] Deletion mutations can also be made in an ST6GalNAcI protein. For example, the mouse ST6GalNAcI protein includes a stem region from about amino acid residues 30-207. Thus, a truncated mouse ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77,  
25 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174,  
30 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, or 207 residues. As another example, the human ST6GalNAcI protein includes a stem region from about amino

acid residues 35-278. Thus, a truncated human ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, or 278 residues. As still another example, chicken ST6GalNAcI protein includes a stem region from about amino acid residues 37-253. Thus, a truncated chicken ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, or 253 residues.

#### D. *One pot refolding of glycosyltransferases*

[0213] These embodiments of the invention are based on the surprising observation that multiple eukaryotic glycosyltransferases expressed in bacterial inclusion bodies can be refolded in a single vessel, *i.e.*, a one pot method. Using this method at least two glycosyltransferases can be refolded together resulting in savings of time and materials.



residues of the yeast ubiquitin gene containing a peptidase cleavage site. Cleavage at the junction of the two moieties results in production of a protein having an intact authentic N-terminal residue.

[0259] The expression vectors of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the *amp*, *gpt*, *neo* and *hyg* genes.

#### VI. Proteins and protein purification

[0260] The recombinant eukaryotic glycosyltransferase proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally, R. Scopes, Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification.*, Academic Press, Inc. N.Y. (1990)). In preferred embodiments, purification of the recombinant eukaryotic glycosyltransferase proteins occurs after refolding of the protein. Substantially pure compositions of at least about 70 to 90%, homogeneity are preferred; more preferably at least 91%, 92%, 93%, 94%, 95%, 96%, or 97%; and 98 to 99% or more homogeneity are most preferred. The purified proteins may also be used, *e.g.*, as immunogens for antibody production.

[0261] To facilitate purification of the recombinant eukaryotic glycosyltransferase proteins of the invention, the nucleic acids that encode the recombinant eukaryotic glycosyltransferase proteins can also include a coding sequence for an epitope or "tag" for which an affinity binding reagent is available, *i.e.* a purification tag. Examples of suitable epitopes include the myc and V-5 reporter genes; expression vectors useful for recombinant production of fusion proteins having these epitopes are commercially available (*e.g.*, Invitrogen (Carlsbad CA) vectors pcDNA3.1/Myc-His and pcDNA3.1/V5-His are suitable for expression in mammalian cells). Additional expression vectors suitable for attaching a tag to the fusion proteins of the invention, and corresponding detection systems are known to those of skill in the art, and several are commercially available (*e.g.*, "FLAG" (Kodak, Rochester NY).

Another example of a suitable tag is a polyhistidine sequence, which is capable of binding to metal chelate affinity ligands. Typically, six adjacent histidines (SEQ ID NO:67) are used,

although one can use more or less than six. Suitable metal chelate affinity ligands that can serve as the binding

enzyme, GST-ST3-GalIII, was active and transferred sialic acid to an LNnT sugar substrate and to asialylated glycoproteins, for example, transferrin and Factor IX.

#### **Cloning ST3GalIII into pGEX-Xt-KT vector**

[0305] Rat liver ST3-GalIII gene was cloned into *Bam*H1 and *Eco*R1 sites of the pGEX-KT-Ext vector after PCR Amplification using the following primers:

Sense Sial 5'Tm 5'-TTTGGATCCAAGCTACACTTACTCCAATGG (SEQ ID NO:68)

Antisense: Sial 3' Whole 5'-TTTGAATTCTCAGATACCACTGCTTAAGTC (SEQ ID NO:69)

#### **Expression of GST-ST3GalIII in *E. coli* BL21 cells**

[0306] pGEX-ST3GalIII, an expression vector comprising the ST3GalIII GST fusion, was transformed into chemically competent *E. coli* BL21 cells. Single colonies were picked, inoculated into five ml LB media with 100 µg/ml carbenicillin, and grown overnight at 37°C with shaking. The next day, one ml of overnight culture was transferred into one liter of LB media with 100 µg/ml carbenicillin. Bacteria were grown until to an OD<sub>620</sub> of 0.7, then 150 µM IPTG (final) was added to the medium. Bacteria were grown at 37°C for one to two hours more, then shifted to room temperature and grown overnight with shaking. Cells were harvested by centrifugation; bacterial pellets were resuspended in PBS buffer and lysed using a French Press. Soluble and insoluble fractions were separated by centrifugation for thirty minutes at 10,000 RPM in a Sorvall, SS 34 rotor at 4°C.

#### **Purification of the inclusion bodies**

[0307] Fifty ml of Novagen's Wash buffer (20 mM Tris.HCl, pH 7.5, 10 mM EDTA, 1 % Triton X-100) was added to the insoluble fraction, *i.e.*, the inclusion bodies (IB's). The insoluble fraction was vortexed to resuspend the pellet. The suspended IB's were centrifuged and washed at least twice by resuspending in Wash Buffer as above. Clean precipitates (IB's) were recovered and were stored at -20 °C until use.

#### **Refolding inclusion bodies**

[0308] The IB's were weighed (144 mg) and dissolved in Genotech IBS buffer (1.44 ml). The resuspended IB's were incubated at 4 °C for one hour in an Eppendorf centrifuge tube. Insoluble material was removed by centrifugation at maximum speed in an Eppendorf centrifuge. Solubilized IB's were diluted to 4 ml final volume. Refolding of GST-ST3GalIII was tested in refolding buffer solutions containing cyclodextrin, polyethylene glycol (PEG),

ND SB-201, or a GSH/GSSG redox system. One ml of solubilized IB's were diluted rapidly by pipetting into the refolding solution, vigorously mixed for 30-40 seconds, and then gently

**Table 3.** GST-ST3GalIII activities after two separate folding experiments using GSH/GSSG system.

<u>GSH/GSSG</u>	<u>Conc</u>	<u>Activity</u>
Refolding Trial 1	12x	182 U/L*
Refolding Trial 2	40x	531 U/L*

\*Activities reported here are Units per L refolded enzyme

#### **Sialylation of glycoproteins using refolded GST-ST3 Gal III**

[0311] Twenty  $\mu$ L of asialylated Transferrin ( $2\mu\text{g}/\mu\text{L}$ ) or asialylated Factor IX ( $2\mu\text{g}/\mu\text{L}$ ), was added to fifty  $\mu$ L of a buffer containing 50mM Tris, pH 8.0; and 150 mM NaCl, with 10  $\mu$ L of 100 mM  $\text{MnCl}_2$ ; 10  $\mu$ L of 200mM CMP-NAN; and 0.05% sodium azide. The reaction mixture was incubated with 30  $\mu$ L refolded GST-ST3GalIII at 30°C overnight or longer with shaking at 250 rpm. After the reactions were stopped, the sialylated proteins were separated on pH 7-3 IEF (Isoelectric focusing gel, Invitrogen) and stained with Coomassie Blue according to manufacturer's guideline. Both Transferrin and Factor IX were sialylated by GST-ST3GalIII. (Data not shown.)

#### *Refolding a rat liver ST3GalIII fused to an MBP tag.*

[0312] Rat liver ST3GalIII was cloned into pMAL-c2x vector and expressed as a maltose binding protein (MBP) fusion, MBP-ST3GalIII, in inclusion bodies of *E.coli* TB1 cells. The refolded MBP-ST3GalIII was active and transferred sialic acid to LNnT, a sugar substrate, and to asialylated glycoproteins, for example asialo-transferrin.

#### **Cloning ST3GalIII into pMAL-c2x vector**

[0313] The rat liver ST3-GalIII nucleic acid was cloned into *Bam*H1 and *Xba*I sites of the pMAL-c2x vector after PCR Amplification using the following primers:

Sense ST3BAMH1 5'-TAATGGATTCAAGCTACACTTACTCCAATGG (SEQ ID NO:70)  
Antisense: ST3XBA1 5'-GCGCTCTAGATCAGATACCACTGCTTAAGT (SEQ ID NO:71)

[0314] Nucleotides encoding amino acids 28-374, *e.g.*, the stem region and catalytic domain of ST3GalIII, were fused to the MBP amino acid tag.

[0315] Three other truncations of ST3GalIII were constructed and fused to MBP. The three ST3Gal III ( $\Delta 73$ ,  $\Delta 85$ ,  $\Delta 86$ ) inserts were isolated by PCR using the following 5'

primers (ST3 BamH1  $\Delta$ 73) TGTATCGGATCCCTGGCCACCAAGTACGCTAACTT (SEQ ID NO:72); (ST3 BamH1  $\Delta$ 85) TGTATCGGATCCTGCAAACCCGGCTACGCTTCAGCCAT (SEQ ID NO:73); and (ST3

BamHI Δ86) TGTATCGGATCCAAACCCGGCTACGCTTCAGCCAT (SEQ ID NO:74) respectively, in pairs with the common 3' primer (ST3-XhoI)GGTCTCCTCGAGTCAGATAACCACTGCTTAA (SEQ ID NO:75). Each PCR product was digested with BamHI and XhoI, subcloned into BamHI-XhoI digested pCWin2-MBP Kanr vector, transformed into TB1 cells, and screened for the correct construct.

[0316] PCR reactions were carried out under the following conditions. One cycle at 95°C for 1 minute. One  $\mu$ l vent polymerase was added. Ten of the following cycles were performed: 94°C for 1 minute; 65°C for 1 minute; and 72°C for 1 minute. After a final ten minutes at 72°C, the reaction was cooled to 4°C.

[0317] All of the ST3GalIII truncations had activity after refolding. The experiments described below were performed using the MBP Δ73ST3GalIII truncation.

#### **Expression of MBP-ST3GalIII in *E. coli* TB1 cells**

[0318] The pMAL-ST3GalIII plasmid was transformed into chemically competent *E. coli* TB1 cells. Three isolated colonies containing TB1/pMAL-ST3GalIII construct were picked from the LB agar plates. The colonies were grown in five ml of LB media supplemented with 60  $\mu$ g/ml carbenicillin at 37°C with shaking until the liquid cultures reached an OD<sub>620</sub> of 0.7. Two one ml aliquots were withdrawn from each culture and used to inoculate fresh media with or without 500  $\mu$ M IPTG (final). The cultures were grown at 37°C for two hours. Bacterial cells were harvested by centrifugation. Total cell lysates were prepared heating the cell pellets in the presence of SDS and DTT. IPTG induced expression of MBP-ST3GalIII. (Data not shown.)

#### **Expression of MBP-ST3GalIII and Purification of the inclusion bodies:**

[0319] A one ml aliquot of TB1/pMAL-ST3GalIII overnight culture was inoculated into 0.5 liter of LB media with 50  $\mu$ g/ml carbenicillin and grown to an OD<sub>620</sub> of 0.7. Expression of MBP-ST3GalIII was induced by addition of 0.5 mM IPTG, followed by overnight incubation at room temperature. The next day bacterial cells were harvested by centrifugation. Cell pellets were resuspended in a buffer containing 75 mM TrisHCl, pH 7.4; 100 mM NaCl; and 1 % glycerol. Bacterial cells were lyzed using a French Press. Soluble and insoluble fractions were separated by centrifugation for thirty minutes, 4°C, 10,000 rpm, Sorvall, SS 34 rotor). Soluble and insoluble fractions were separated by centrifugation for thirty minutes at 10,000RPM in a Sorvall, SS 34 rotor at 4°C.

buffer was supplemented with 0.3 mM Lauryl maltoside (LM); 0.1 mM oxidized glutathione (GSSG); 1 mM reduced glutathione (GSH) immediately before the addition of solubilized IB's. Two ml of solubilized IB's were added into 43 ml of refolding buffer in 50 ml sterile culture tube. The tube was placed on a rocker-shaker and gently shaken for 24 hours at 4°C.

- 5 The refolded protein was dialyzed in dialysis tubing ( MWCO: 7 kD) against Dialysis Buffer (100 mM Tris HCl, pH 7.5; 100 mM NaCl; and 5 % glycerol) twice (in 10-20 volume excess buffer).

[0330] The large scale dialyzed, refolded MBP-Gal III was analyzed for ST3GalIII activity, and exhibited about 53.6 U/g IB.

10 Example 2: Site Directed Mutagenesis of Human GnTI to Enhance Refolding.

[0331] A truncated human N-acetylglucosaminyltransferase I (103 amino terminal amino acids deleted) was expressed in *E.coli* as a maltose binding fusion protein (GnTI/MBP). The fusion protein was insoluble and was expressed in inclusion bodies. After solubilization and refolding, the GnTI/MBP fusion protein had low activity. The crystal structure of a truncated  
15 form of rabbit GnTI (105 amino terminal amino acids deleted) shows an unpaired cysteine residue (CYS123) near the active site. (See, *e.g.*, Unligil *et al.*, *EMBO J.* 19:5269-5280 (2000)). The corresponding unpaired cysteine in the human GnTI was identified as CYS121 and was replaced with a series of amino acids that are similar in size and chemical characteristics. The amino acids used include serine (Ser), threonine (Thr), alanine (Ala) and  
20 aspartic acid (Asp). In addition, a double mutant, ARG120ALA, CYS121HIS, was also made. The mutant GnTI/MBP fusion proteins were expressed in *E. coli*, refolded and assayed for GnTI activity towards glycoproteins.

[0332] Mutagenesis was done using a Quick Change Site-Directed Mutagenesis Kit from Stratagene. Additional restriction sites were introduced with some of the GnTI mutations.

- 25 For example an *ApaI* site (underlined, GGGCCCAC) was introduced into the GnTI ARG120ALA, CYS121HIS mutant, *i.e.*, CGC CTG → **GCC CAC** (changes in bold). The following mutagenic oligonucleotides were used to make the double mutant: GnTI R120A, C121H+, 5'CCGCAGCACTGTTCGGGCCACCTGGACAAGCTGCTG 3' (SEQ ID NO:76); and GnTI R120A, C121H-  
30 5'CAGCAGCTTGTCCAGGTGGGCCCGAACAGTGCTGCGG 3' (SEQ ID NO:77) (changes shown in bold). An *AscI* site (underlined, GGCGCGCC) was introduced into the



GnT1 CYS121ALA mutant, *i.e.*, CTG → **GCC** (changes in bold). The following mutagenic oligonucleotides were used to make the GnT1 CYS121ALA mutant: GnT1C123A+

5'AGCACTGTTTCGGCGCGCCCTGGACAAGCTGCTG 3' (SEQ ID NO:78); and  
GnT1C123A-5'CAGCAGCTTGTCCAGGGCGCGCCGAACAGTGCT 3' (SEQ ID  
NO:79).

[0333] The activity of the mutant proteins expressed in *E. coli* was compared to the activity  
5 of wild type GnT1 expressed in baculovirus. A CYS121SER GNTI mutant was active in a  
TLC based assay. In contrast, a CYS121THR mutant had no detectable activity and a  
CYS121ASP mutant had low activity. A CYS121ALA mutant was very active, and a double  
mutant, ARG120ALA, CYS121HIS, based on the amino acid sequence of the *C. elegans*  
GnT1 protein (Gly14), also exhibited activity, including transfer of GlcNAc to glycoproteins.  
10 Amino acid and encoding nucleic acid sequences of the GnT1 mutants are provided in  
Figures 7-11.

[0334] A second GnT1 truncation was made and fused to MBP: MBP-GnT1(D35). Figure  
35 provides a schematic of the MBP-GnT1 fusion proteins, and depicts the truncations, e.g.,  
Δ103 or Δ35, and the Cys121Ser mutation (top). The bottom of the figure provides the full  
15 length human GnT1 protein. Mutations of Cys121 were also made in the MBP-GnT1(D35)  
protein.

[0335] Both fusion proteins were expressed in *E. coli* and both had activity for remodeling  
of the RNase B glycoprotein. Figure 36 provides an SDS-PAGE gel showing in the right  
panel the refolded MBP-GnT1 fusion proteins: MBP-GnT1(D35) C121A, MBP-GnT1(D103)  
20 R120A + C121H, and MBP-GnT1(D103) C121A. The left panel shows the activities for  
remodeling the RNase B glycoprotein of two different batches (A1 and A2) of refolded  
MBP-GnT1(D35) C121A at different time points. The MBP-GnT1 (D103) C121A also  
remodeled the RNase B glycoprotein. Data not shown.

#### Example 3: MPB fusions to GalT1.

25 [0336] The following fusions between truncated bovine GalT1 and MBP were constructed:  
MBP-GalT1 (D129) wt, (D70) wt or (D129 C342T). (For the full length bovine sequence,  
see, e.g., D'Agostaro *et al.*, *Eur. J. Biochem.* 183:211-217 (1989) and accession number  
CAA32695.) Each construct had activity after refolding. The amino acid sequence of the  
full length bovine GalT1 protein is provided in Figure 30. The mutants are depicted  
30 schematically in Figure 31 with a control protein GalT1(40) (S96A+C342T). See, e.g.,  
Ramakrishnan *et al.*, *J. Biol. Chem.* 276:37666-37671 (2001).

**[0337]** MBP-GalT1 (D70) was expressed in *E. coli* strain JM109. After overnight induction with IPTG, inclusion bodies were isolated from the insoluble pellet after cells were

Example 5: Refolding eukaryotic GalNAcT2.

[0365] A truncated human GalNAcT2 enzyme was expressed in *E. coli* and used to determine optimal conditions for solubilization and refolding using the methods described above. The full length human GalNAcT2 nucleic acid and amino acid sequences are provided in Figures 13A and B. The sequences of the mutant protein, GalNAcT2(D51), are shown in Figures 14A and B. The mutant was expressed in *E. coli* as an MBP fusion protein, MBP-GalNAcT2(D51). Other GalNAcT2 mutants were made, expressed in *E. coli* and were able to be refolded: MBP-GalNAcT2(D40), MBP-GalNAcT2(D73), and MBP-GalNAcT2(D94). Data not shown. Details of the construction of the additional deletion mutants is found in USSN 60/576,530, filed June 3, 2004 and USSN 60/598,584, August 3, 2004, both of which are herein incorporated by reference for all purposes.

[0366] Cultures of bacteria expressing MBP-GalNAcT2(D51) were grown and harvested as described above. Inclusion bodies were purified from bacteria as described above. Solubilization of the inclusion bodies was performed at pH 6.5 or at pH 8.0. After solubilization, MBP-GalNAcT2(D51) protein was refolded at either pH 6.5 or pH 8.0 using buffers A and B, *i.e.*, **Buffer A**: 55 mM MES pH 6.5, 550 mM Arginine, 0.055 % PEG3350, 264 mM NaCl, 11 mM KCl, supplemented with 1 mM GSH, 0.1 mM GSSG; and **Buffer B**: 55 mM TrisHCl pH 8, 550 mM Arginine, 0.055 % PEG3350, 264 mM NaCl, 11 mM KCl, supplemented with 1 mM GSH, 0.1 mM GSSG. After refolding, MBP-GalNAcT2(D51) protein was dialyzed and then concentrated. Figure 15 provides a demonstration of the protein concentration of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0.

[0367] A radiolabeled [<sup>3</sup>H]-UDP-GalNAc assay was performed to determine the activity of the *E. coli*-expressed refolded MBP-GalNAcT2(D51) by monitoring the addition of radiolabeled GalNAc to a peptide acceptor. The acceptor was a MuC-2 – like peptide having the sequence MVTPTPTPTC (SEQ ID NO:80). The peptide was dissolved in 1M Tris-HCl pH=8.0. See, *e.g.*, USSN 60/576,530 filed June 3, 2004; and US provisional patent application Attorney Docket Number 040853-01-5149-P1, filed August 3, 2004; both of which are herein incorporated by reference for all purposes. Figure 16 provides a demonstration of the enzymatic activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. Figure 17 provides a demonstration of the specific activity of refolded MBP-GalNAcT2(D51) after solubilization

at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The highest activity levels were observed with MBP-GalNAcT2(D51) that

## Human ST6GalNAcTI

MRSCWRCRHLSSQGVQWSLLAVLVFFLFALPSFIKEPQTKPSRHQRTENIKERSLQS  
 LAKPKSQAPTRARRTTIYAEPVPENNALNTQTQPKAHTTGDRGKEANQAPPEEQDK  
 VPHTAQRAAWKSPEKEKTMVNTLSPRGQDAGMASGRTEAQSWSQDTKTTQNGG  
 GQTRKLTASRTVSEKHQGAATTAKTLIPKSQHRMLAPTGAVSTRTRQKGVTTAVIP  
 PKEKKPQATPPPAPFQSPTTQRNQRLLKAANFKSEPRWDFEEKYSFEIGGLQTTCPDSV  
 KIKASKSLWLQKLFLPNLTLFLDSRHFNQSEWDRLEHFAPPFGFMELNYSLVQKVVT  
 RFPPVPQQQLLLASLPAGSLRCITCAVVGNGGILNNSHMGQEIDSHDYVFRLSGALIK  
 GYEQDVGTRTSFYGFTAFSLTQSLILGNRGFKNVPLGKDVRYLHFLEGTRDYEWLE  
 ALLMNQTVMSKNLFWFRHRPQEAFFREALHMDRYLLHPDFLRYMKNRFLRSKTLT  
 GAHWRIYRPTTGALLLLTALQLCDQVSAYGFITEGHERFSDHYDTSWKRLIFYINH  
 DFKLEREVWKRLHDEGIIRLYQRPGPGTAKAKN

**FIG. 38A**

## Chicken ST6GalNAcTI

MGFLIRRLPKDSRIFRWLLILTVFSFIITSFSALFGMEKSIFRQLKIYQSIAHMLQVDTQ  
 DQQGSNYSANGRISKVGLERDIAWLELNTAVSTPSGEGKEEQKKTVPKPAKVEEAK  
 EKVTVPKPFPEVMGITNTTASTASVVERTKEKTTARPVPGVGEADGKRTTIALPSMKE  
 DKEKATVKPSFGMKVAHANSTSKDKPKAEPPASVKAIRPVTQAATVTEKKKLRAA  
 DFKTEPQWDFDDEYILDSSSPVSTCSESVRAKAAKSDWLRDLFLPNITLFDKSYFNV  
 SEWDRLEHFAPPYGFMEELNYSLVVEEVMRLPPNPHQQLLLANSSSNVSTCISCAVVG  
 NGGILNNSGMGQEIDSHDYVFRVSGAVIKGYEKDVGKTSFYGFTAYSLSVSSLQNLG  
 HKGFKKIPQGKHIRYIHFLAVRDYEWLKAALLDKDIRKGFLNYYGRRPRERFDEDF  
 TMNKYLVAHPDFLRYLKNRFLKSKNLQKPYWRLYRPTTGALLLLTALHLCDRVSAY  
 GYITEGHQKYSDDHYDKEWKRLVFYVNHDFNLEKQVWKRLHDENIMKLYQRS

**FIG. 38B**

## Mouse ST6GalNAcTI protein beginning at residue 32 of the native mouse protein

DPRAKDSRCQFIWKNDASAEQENQQAEPQVPIMTLSPRVHNKESTSVSSKDLKKQER  
 EAVQGEQAEGKEKRKLETIRPAPENPQSKAEPAAKTPVSEHLDKLPRTPGALSTRKTP  
 MATGAVPAKKKVVQATKSPASSPHPTTRRRQRLKASEFKSEPRWDFEEYSLDMSSL  
 QTNCASAVKIKASKSPWLQNIFLPNITLFLDSGRFTQSEWNRLEHFAPPFGFMELNQSL  
 VQKVVTFRPPVRQQQLLLASLPTGYSKCITCAVVGNGGILNDSRVGREIDSHDYVFR  
 LSGAVIKGYEQDVGTRTSFYGFTAFSLTQSILILGRRGFQHVPLGKDVRYLHFLEGTR  
 NYEWLEAMFLNQTLAKTHLSWFRHRPQEAFFRNALDLRYLLHPDFLRYMKNRFL  
 RSKTLDTAHWRIYRPTTGALLLLTALHLCDKVSAYGFITEGHQRFSDHYDTSWKRL  
 IFYINHDFRLERMVWKRLHDEGIHWLYQRQPSDKAKN

**FIG. 38C**

	1		50
HSGALNAT1.pep	(1)MRKFAYCKVVLATSLIWVLLDMFLLLYFS-----	ECNKC	
HSGALNAT2.pep	(1)MRRRS--RMLLCFAFLWVLGIAYMYSGGGSALAGGAGGAGRKEDWNEI		
Consensus	(1)MRK A KMLL A IWVL F L	D N	
	51		100
HSGALNAT1.pep	(35)DEKKERGLPAGDVLEPVQKPHEGP-G-----	EMGKPVVIPKEDQEKMKEM	
HSGALNAT2.pep	(49)DPIKKKDLHHSNGEKAQSMETLPPGKVRWPDFNQEAYVGGIMVRSQDP		
Consensus	(51)D K K L E Q P G D I D		
	101		150
HSGALNAT1.pep	(79)FKINQFNLMASEMIALNRS�PDVRLEGCKTKVYPDNLPITTSVVTVFHNEA		
HSGALNAT2.pep	(99)YARNKFNQVESDKLRMDRAIPDTRHDQQRKQWRVDLPATSVVITFHNEA		
Consensus	(101)F N FN M SD I L RAIPD R D C K W LP TSVVI FHNEA		
	151		200
HSGALNAT1.pep	(129)WSTLLRTVHSVINRSPRHMIETVLVDDASERDFLKRPLESYVKKLKVPV		
HSGALNAT2.pep	(149)RSALLRTVVSVLKKSPPHLIKEIILVDDYSN-----	DPEDGALLGKIEKV	
Consensus	(151) S LLRTV SVI KSP HLI EIIIVDD S P D L V		
	201		250
HSGALNAT1.pep	(179)HVIRMEQSRGLIRARLKGA AVSKGQVITFLDAH <b>Ce</b> CTVGWLEPLLARIKH		
HSGALNAT2.pep	(194)RVLNRDRREGLMRSRVRGADAAQAKVLTFLDSH <b>Ce</b> CNEHWLEPLLervae		
Consensus	(201) VIR D R GLIRARLKGA A A VITFLDAH <b>CeC</b> WLEPLL RI		
	251		300
HSGALNAT1.pep	(229)DRRTVVCPIIDVISDDTFEYMAGSDMTYGGFNWKLNFRWYPVPQREMDRR		
HSGALNAT2.pep	(244)DRTRVVSPIIDVINMDNFQYVGASADLKGGFWDNLVFKWDYMTPEQRRSR		
Consensus	(251) DR VV PIIDVI D F YMAAS GGF W L FKW M R		
	301		350
HSGALNAT1.pep	(279)KGDRTLVPVRIPIMAGGLFSIDRDYFQEIGTYDAGMDIWGGENLEISFRIW		
HSGALNAT2.pep	(294)QGNPVAPIKTEMIAGGLFVMDKFYFEELGKYDMMMDVWGENLEISFRVW		
Consensus	(301) G PIKTP IAGGLF IDK YF EIG YD MDIWGGENLEISFRIW		
	351		400
HSGALNAT1.pep	(329)QCGGTLEIVICSHVGHVFRKATPYTFPGGIGQIINKNNRRRLAEVWMDEFK		
HSGALNAT2.pep	(344)QCGGSLEIIPCSRVGHVFRKQHPYTFPGSGTVFARNTRRAAEVWMDEYK		
Consensus	(351) QCGGSLEII CS VGHVFRK PYTFPGSG I KN RR AEVWMDEFK		
	401		450
HSGALNAT1.pep	(379)NFFYIISPQVTKVDYGDISSRVGLRHKLQCKPFSWYLENTYPDSQIPRHY		
HSGALNAT2.pep	(394)NFFYYAAVPSARNVPYGNIQSRLELRKKLSCKPFWYLENVYPELRVPDHQ		
Consensus	(401) NFFY P V YG I SRL LR KL CKPF WYLENTYPD IP H		
	451		500
HSGALNAT1.pep	(429)FSLGEIRNVEINQCLDNMARKENKVGIFNCHGMGGNQVFSYTANKEIRT		
HSGALNAT2.pep	(444)DIAFGALQQGIN-CLDITLGHFADGVVGVYECHNAGGNQEWALITKEKSVKH		
Consensus	(451) N TN CLD LA VGIF CH GGNQ FA T K IK		
	501		550
HSGALNAT1.pep	(479)DDLCLDVSKLN--GPVIMLKCHHLKGNQLWEYDPVKLTQLQHVNSNQCLDK		
HSGALNAT2.pep	(493)MDLCLTVVDRAPGSLIKLQGCRENDSRQKWEQIEGNSKL RHVGSNLCLDS		
Consensus	(501) DLCL V I L C Q WE L HV SN CLD		
	551		584
HSGALNAT1.pep	(527)ATEEDSQVPSIRDNCGRSQQWLLRNVTLPETF-		
HSGALNAT2.pep	(543)RTAK-SGGLSVEVCGPALSQQWKFTLNLQQ----		
Consensus	(551) T S SI C A SQQW		

FIG. 44

[0061] Figure 5 provides the results of an assay of GlycoPEGylation of EPO using the refolded SuperGlycoMix. Lanes are as follows: (1) MW markers, SeeBlue2 Invitrogen,(250, 148, 98, 64, 50, 36, 22, 16, 6 kD); (2) Positive control with EPO, + NSO expressed GalT1, BV GnT1, Aspergillus ST3GalIII and sugar nucleotides; (3) Negative control, Same as 2 without UDP-GlcNAc; (4) EPO, Purified and separately refolded MBP-GalT1(Δ129) C342T, Refolded MBP-GnT1(Δ103), and *Aspergillus niger* expressed ST3GalIII; (5) EPO, SuperGlycoMix (mixture of MBP-ST3GalIII, MBP-GalT1(Δ129) C342T, MBP-GnT1(Δ103)C123A and sugar nucleotides.

[0062] Figure 6 provides an alignment of a human GnT1 amino acid sequence (top line, NP\_002397) and a rabbit GnT1 amino acid sequence (bottom line, P27115). The conserved unpaired cysteines are underlined and in bold text.

[0063] Figure 7 provides the amino acid sequence of a GnT1 Cys121Ser mutant and a nucleic acid sequence that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrldkllh... where the bold residue is mutated from the wild-type cysteine.

[0064] Figure 8 provides the amino acid sequence of a GnT1 Cys121Asp mutant and a nucleic acid sequence that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrldkllh... where the bold residue is mutated from the wild-type cysteine.

[0065] Figure 9 provides the amino acid sequence of a GnT1 Cys121Thr mutant and a nucleic acid sequence that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrldkllh... where the bold residue is mutated from the wild-type cysteine.

[0066] Figure 10 provides the amino acid sequence of a GnT1 Cys121Ala mutant and a nucleic acid sequence that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following unpaired cysteine mutation: ...stvrrldkllh... where the bold residue is mutated from the wild-type cysteine.



(SEQ ID No:15)  
[0067] Figure 11 provides the amino acid sequence of a GnT1 Arg120Ala, Cys121His mutant and a nucleic acid sequence (SEQ ID No:16) that encodes the mutant GnT1 protein. The amino acid sequence depicted begins with amino acid residue 104 of the full length human protein and is representative of mammalian GnT1 proteins with the following double mutation:

5 ...stvr<sup>(SEQ ID No:17)</sup>ahldkllh...<sub>λ</sub> where the bold residue is mutated from the wild-type cysteine.

(SEQ ID No:18)  
[0068] Figure 12 provides the amino acid sequence of rat liver ST3GalIII<sub>λ</sub>. The underlined and italicized sequence was deleted to make the Δ28 deletion.

(SEQ ID No:20) (SEQ ID No:19)  
[0069] Figures 13A and 13B provide full length nucleic acid<sub>λ</sub> and amino acid<sub>λ</sub> sequences of UDP-N-acetylgalactosaminyltransferase 2 (GalNAcT2). The accession number of the  
10 nucleic acid and protein is NM\_004481.

(SEQ ID No:22) (SEQ ID No:21)  
[0070] Figures 14A and 14B provide nucleic acid<sub>λ</sub> and amino acid<sub>λ</sub> sequences of a Δ51GalNAcT2. The numbering is based on the full length amino acid and nucleic acid sequences shown in Figures 13A and B.

[0071] Figure 15 provides a demonstration of the protein concentration of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Protein concentrations were measured immediately after refolding (light gray bars), after dialysis (dark gray bars), and after concentration (white bars).  
15

[0072] Figure 16 provides a demonstration of the enzymatic activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Activity was measured after dialysis (light gray bars) and after concentration (dark gray bars).  
20

[0073] Figure 17 provides a demonstration of the specific activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The pH values tested are expressed as solubilization pH-refolding pH. Specific activity was measured after dialysis (white bars) and after concentration (dark gray bars).  
25

[0074] Figures 18A and 18B provide results of remodeling of recombinant granulocyte colony stimulating factor (GCSF) using refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. Figure 18A shows the results using a control purified MBP-GalNAcT2(D51), or a negative control that lacked a substrate, or  
30

bacterially expressed MBP-GalNAcT2(D51) that was solubilized at pH 6.5 and refolded at pH 6.5. Figure 18B shows the experimental results.

[0075] Figure 19 provides a profile of refolded MBP-GalNAcT2(D51) proteins after elution from a Q Sepharose XL (QXL) column (Amersham Biosciences, Piscataway, NJ).

5 The top of the figure shows a chromatogram illustrating the elution of MBP-GalNAcT2(D51) from the QXL column. Fraction numbers are indicated on the X-axis and the relative absorbance of each fraction is indicated on the Y-axis. The bottom shows an image of two electrophoretic gels used to visualize the eluted fractions. The contents of each lane on the gel are described in the figure.

10 [0076] Figure 20 provides the GalNAcT2 activity of specific column fractions from the QXL column shown in Figure 19. The most active fractions were applied to a Hydroxyapatite Type I (80µm) (BioRad, Hercules, CA) column.

[0077] Figure 21 provides a profile of refolded MBP-GalNAcT2(D51) proteins after elution from the HA type I column. The top of the figure shows a chromatogram illustrating  
15 the elution of MBP-GalNAcT2(D51) from the HA type I column. Fraction numbers are indicated on the X-axis and the relative absorbance of each fraction is indicated on the Y-axis. The bottom shows an image of an electrophoretic gel used to visualize the eluted fractions. The contents of each lane on the gel are described in the figure.

[0078] Figure 22 provides the GalNAcT2 activity of HA type I eluted fractions.

20 [0079] Figure 23 provides a comparison of purification and activity of ST3Gal3 proteins fused to either an MBP tag or to an MBP-SBD tag.

[0080] Figure 24 provides the amino acid sequences of the MBP-ST3Gal1 fusion protein<sub>A</sub> (A) and the MBP-SBD-ST3Gal1 fusion protein<sub>A</sub> (B).  
(Seq ID No: 23)  
(Seq ID No: 24)

[0081] Figure 25 provides the sialyltransferase activity of the MBP-ST3Gal3 fusion  
25 protein) and the MBP-SBD-ST3Gal3 fusion protein. positive and negative controls are also shown.

[0082] Figure 26 provides the amino acid sequence of mouse and human ST6GalNAcI  
proteins fused to MBP. Part A shows the sequence of a mouse truncation fusion: MBP-  
mST6GalNAcI S127<sub>A</sub> (Seq ID No: 25) Part B shows the sequence of a human truncation fusion: MBP-  
hST6GalNAcI K36<sub>A</sub> (Seq ID No: 26)  
30

- [0083] Figure 27 provides SDS-PAGE gels of O-linked glycosyltransferase enzyme (A) concentrations after co-refolding and the (B) results of an enzyme assay after co-refolding. MBP-GalNAcT2 and MBP-ST3GalI were co-refolded together. Enzyme activity was tested after addition of Core I Gal T1 enzyme. The substrates were IFa-2b and 20K-Peg-CMP-NAN.
- [0084] Figure 28 provides an SDS-PAGE gel showing expression of the native SiaA protein in *E. coli* before and after induction with IPTG.
- [0085] Figure 29 provides an SDS-PAGE gel showing expression of an MBP-SiaA fusion protein in *E. coli* before and after induction with IPTG.
- 10 [0086] Figure 30 provides the amino acid sequence of the full length bovine GalT1 protein. <sup>(See ID No:27)</sup>
- [0087] Figure 31 depicts GalT1 mutants schematically, as well as a control protein GalT1(40) (S96A+C342T).
- [0088] Figure 32 provides the results of enzymatic assays of the refolded and purified MBP-GalT1 (D70) protein. The assay measured conversion of LNT2 (Lacto-N-Triose-2) into LNnT (Lacto-N-Neotetraose) using UDP-Gal (Uridine 5'-Diphosphogalactose) as a donor substrate.
- 15 [0089] Figure 33 provides an RNase B remodeling assay of MBP-GalT1 (D70) and a control protein GalT1(40) (S96A+C342T), also referred to as Qasba's GalT1.
- [0090] Figure 34 provides kinetics of glycosylation of RNase B using the refolded and purified MBP-GalT1 (D70) protein or NSO GalT1, a soluble form of the bovine GalT1 protein that was expressed in a mammalian cell system.
- 20 [0091] Figure 35 provides a schematic of the MBP-GnT1 fusion proteins, and depicts the truncations, e.g., Δ103 or Δ35, and the Cys121Ser mutation (top). The bottom of the figure provides the full length human GnT1 protein. <sup>(See ID No:1)</sup>
- 25 [0092] Figure 36 provides an SDS-PAGE gel showing in the right panel the refolded MBP-GnT1 fusion proteins: MBP-GnT1(D35) C121A, MBP-GnT1(D103) R120A + C121H, and MBP-GnT1(D103) C121A. The left panel shows GnT1 activities of two different batches (A1 and A2) of refolded MBP-GnT1(D35) C121A at different time points.
- [0093] Figure 37 provides a full length sequence of porcine ST3GalI. <sup>(See ID No:25)</sup>

[0094] Figure 38 provides full length amino acid sequences for A) human ST6GalNAcI<sub>AA</sub><sup>T</sup> (Seq ID No: 29) and for B) chicken ST6GalNAcI<sub>AA</sub><sup>T</sup> (Seq ID No: 30) and C) a sequence of the mouse ST6GalNAcI<sub>AA</sub><sup>T</sup> protein beginning at residue 32 of the native mouse protein<sub>AA</sub> (Seq ID No: 31)

[0095] Figure 39 provides a schematic of a number of preferred human ST6GalNAcI truncation mutants.

[0096] Figure 40 shows a schematic of MBP fusion proteins including the human ST6GalNAcI truncation mutants.

[0097] Figure 41 provides the full length sequence of human Core 1 GalT1 protein<sub>AA</sub> (Seq ID No: 32)

[0098] Figure 42 provides the sequences of two Drosophila Core 1 GalT1 proteins<sub>AA</sub> (Seq ID Nos: 33 and 34)

10 [0099] Figure 43 provides the sequences of exemplary bacterial MBP proteins that can be fused to glycosyltransferases to enhance refolding. A. *Yersinia* MBP<sub>AA</sub> (Seq ID No: 35); B. *E. coli* MBP<sub>AA</sub> (Seq ID No: 36); C. *Pyrococcus furiosus* MBP<sub>AA</sub> (Seq ID No: 37); D. *Thermococcus litoralis* MBP<sub>AA</sub> (Seq ID No: 38); E. *Thermatoga maritime* MBP<sub>AA</sub> (Seq ID No: 39) and F. *Vibrio cholerae* MBP<sub>AA</sub> (Seq ID No: 40)

[0100] Figure 44 provides an alignment of human GalNAcT1 and GalNAcT2 proteins<sub>AA</sub> (Seq ID No: 41) (Seq ID No: 42)

15 Because the alignment programs account for sequence insertions or deletions, the numbering of cysteine residues is not the same as mentioned text and published sequences. In the case of hGalNAc-T2 cysteine 227 (published) corresponds to position 235 in the alignment and cysteine 229 (published) is 237 in the alignment. The hGalNAc-T1 cysteines are 212 (published), which corresponds to cysteine 235 (alignment) and 214 (published), which  
20 corresponds to cysteine 237 (alignment). The relevant cysteine residues are indicated by larger font size. Consensus peptides = Seq ID Nos: 42-65.

[0101] Figure 45 shows the position of paired and unpaired cysteine residues in the human ST6GalNAcI protein. Single and double cysteine substitution are also shown, e.g., C280S, C362S, C362T, (C280S + C362S), and (C280S + C362T).

## 25 DEFINITIONS

[0102] The recombinant glycosyltransferase proteins of the invention are useful for transferring a saccharide from a donor substrate to an acceptor substrate. The addition generally takes place at the non-reducing end of an oligosaccharide or carbohydrate moiety on a biomolecule. Biomolecules as defined here include but are not limited to biologically

[0133] A "fusion protein" refers to a protein comprising amino acid sequences that are in addition to, in place of, less than, and/or different from the amino acid sequences encoding the original or native full-length protein or subsequences thereof.

[0134] Components of fusion proteins include "accessory enzymes" and/or "purification tags." An "accessory enzyme" as referred to herein, is an enzyme that is involved in catalyzing a reaction that, for example, forms a substrate for a glycosyltransferase. An accessory enzyme can, for example, catalyze the formation of a nucleotide sugar that is used as a donor moiety by a glycosyltransferase. An accessory enzyme can also be one that is used in the generation of a nucleotide triphosphate required for formation of a nucleotide sugar, or in the generation of the sugar which is incorporated into the nucleotide sugar. The recombinant fusion protein of the invention can be constructed and expressed as a fusion protein with a molecular "purification tag" at one end, which facilitates purification of the protein. Such tags can also be used for immobilization of a protein of interest during the glycosylation reaction. Suitable tags include "epitope tags," which are a protein sequence that is specifically recognized by an antibody. Epitope tags are generally incorporated into fusion proteins to enable the use of a readily available antibody to unambiguously detect or isolate the fusion protein. A "FLAG tag" is a commonly used epitope tag, specifically recognized by a monoclonal anti-FLAG antibody, consisting of the sequence  
(SEQ ID NO: 66)  
AspTyrLysAspAspAsp<sup>^</sup>AspLys<sub>A</sub> or a substantially identical variant thereof. Other suitable tags are known to those of skill in the art, and include, for example, an affinity tag such as a hexahistidine peptide<sub>A</sub> (SEQ ID NO: 67) which will bind to metal ions such as nickel or cobalt ions. Proteins comprising purification tags can be purified using a binding partner that binds the purification tag, e.g., antibodies to the purification tag, nickel or cobalt ions or resins, and amylose, maltose, or a cyclodextrin. Purification tags also include starch binding domains, *E. coli* thioredoxin domains (vectors and antibodies commercially available from e.g., Santa Cruz Biotechnology, Inc. and Alpha Diagnostic International, Inc.), and the carboxy-terminal half of the SUMO protein (vectors and antibodies commercially available from e.g., Life Sensors Inc.). Maltose binding domains are preferably used for their ability to enhance refolding of insoluble eukaryotic glycosyltransferases, but can also be used to assist in purification of a fusion protein. Purification of maltose binding domain proteins is known to those of skill in the art. Starch binding domains are described in WO 99/15636, herein incorporated by reference. Affinity purification of a fusion protein comprising a starch binding domain using

about amino acid residues 32-90. Thus, a truncated human Core1 GalT1 protein can have deletions at the amino terminus of about *e.g.*, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 residues.

- 5 [0210] Deletion mutations can also be made in an ST3Gal1 protein. For example, the human ST3Gal1 protein includes a stem region from about amino acid residues 18-58. Thus, a truncated human ST3Gal1 protein can have deletions at the amino terminus of about *e.g.*, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, or 58 residues. As another example, the
- 10 porcine ST3Gal1 protein includes a stem region from about amino acid residues 28-61. Thus, a truncated porcine ST3Gal1 protein can have deletions at the amino terminus of about *e.g.*, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, or 61 residues.

- [0211] Deletion mutations can also be made in a GalNAcT2 protein. For example, the rat
- 15 GalNAcT2 protein includes a stem region from about amino acid residues 40-95. Thus, a truncated rat GalNAcT2 protein can have deletions at the amino terminus of about *e.g.*, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 residues.

- 20 [0212] Deletion mutations can also be made in an ST6GalNAcI protein. For example, the mouse ST6GalNAcI protein includes a stem region from about amino acid residues 30-207. Thus, a truncated mouse ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77,
- 25 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174,
- 30 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, or 207 residues. As another example, the human ST6GalNAcI protein includes a stem region from about amino

acid residues 35-278. Thus, a truncated human ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, or 278 residues. As still another example, chicken ST6GalNAcI protein includes a stem region from about amino acid residues 37-253. Thus, a truncated chicken ST6GalNAcI protein can have deletions at the amino terminus of about *e.g.*, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, or 253 residues.

#### D. *One pot refolding of glycosyltransferases*

[0213] These embodiments of the invention are based on the surprising observation that multiple eukaryotic glycosyltransferases expressed in bacterial inclusion bodies can be refolded in a single vessel, *i.e.*, a one pot method. Using this method at least two glycosyltransferases can be refolded together resulting in savings of time and materials.

residues of the yeast ubiquitin gene containing a peptidase cleavage site. Cleavage at the junction of the two moieties results in production of a protein having an intact authentic N-terminal residue.

5 [0259] The expression vectors of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the *amp*, *gpt*, *neo* and *hyg* genes.

#### VI. Proteins and protein purification

10 [0260] The recombinant eukaryotic glycosyltransferase proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see, generally, R. Scopes, Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in Enzymology Vol. 182: Guide to Protein Purification.*, Academic Press, Inc. N.Y. (1990)). In preferred  
15 embodiments, purification of the recombinant eukaryotic glycosyltransferase proteins occurs after refolding of the protein. Substantially pure compositions of at least about 70 to 90%, homogeneity are preferred; more preferably at least 91%, 92%, 93%, 94%, 95%, 96%, or 97%; and 98 to 99% or more homogeneity are most preferred. The purified proteins may also be used, *e.g.*, as immunogens for antibody production.

20 [0261] To facilitate purification of the recombinant eukaryotic glycosyltransferase proteins of the invention, the nucleic acids that encode the recombinant eukaryotic glycosyltransferase proteins can also include a coding sequence for an epitope or "tag" for which an affinity binding reagent is available, *i.e.* a purification tag. Examples of suitable epitopes include the myc and V-5 reporter genes; expression vectors useful for recombinant production of fusion  
25 proteins having these epitopes are commercially available (*e.g.*, Invitrogen (Carlsbad CA) vectors pcDNA3.1/Myc-His and pcDNA3.1/V5-His are suitable for expression in mammalian cells). Additional expression vectors suitable for attaching a tag to the fusion proteins of the invention, and corresponding detection systems are known to those of skill in the art, and several are commercially available (*e.g.*, "FLAG" (Kodak, Rochester NY).

30 Another example of a suitable tag is a polyhistidine sequence, which is capable of binding to metal chelate affinity ligands. Typically, six adjacent histidines<sup>(see ID No. 67)</sup> are used, although one can use more or less than six. Suitable metal chelate affinity ligands that can serve as the binding



enzyme, GST-ST3-GalIII, was active and transferred sialic acid to an LNnT sugar substrate and to asialylated glycoproteins, for example, transferrin and Factor IX.

#### Cloning ST3GalIII into pGEX-XT-KT vector

[0305] Rat liver ST3-GalIII gene was cloned into *Bam*H1 and *Eco*R1 sites of the pGEX-

5 KT-Ext vector after PCR Amplification using the following primers:

Sense Sial 5'Tm 5'-TTTGGATCCAAGCTACACTTACTCCAATGG<sup>(Seq. ID No: 68)</sup>  
Antisense: Sial 3' Whole 5'-TTTGAATTCTCAGATACCACTGCTTAAGTC<sup>(Seq. ID No: 69)</sup>

#### Expression of GST-ST3GalIII in *E. coli* BL21 cells

10 [0306] pGEX-ST3GalIII, an expression vector comprising the ST3GalIII GST fusion, was transformed into chemically competent *E. coli* BL21 cells. Single colonies were picked, inoculated into five ml LB media with 100 µg/ml carbenicillin, and grown overnight at 37°C with shaking. The next day, one ml of overnight culture was transferred into one liter of LB media with 100 µg/ml carbenicillin. Bacteria were grown until to an OD<sub>620</sub> of 0.7, then 150  
15 µM IPTG (final) was added to the medium. Bacteria were grown at 37°C for one to two hours more, then shifted to room temperature and grown overnight with shaking. Cells were harvested by centrifugation; bacterial pellets were resuspended in PBS buffer and lysed using a French Press. Soluble and insoluble fractions were separated by centrifugation for thirty minutes at 10,000 RPM in a Sorvall, SS 34 rotor at 4°C.

#### 20 Purification of the inclusion bodies

[0307] Fifty ml of Novagen's Wash buffer (20 mM Tris.HCl, pH 7.5, 10 mM EDTA, 1 % Triton X-100) was added to the insoluble fraction, *i.e.*, the inclusion bodies (IB's). The insoluble fraction was vortexed to resuspend the pellet. The suspended IB's were centrifuged and washed at least twice by resuspending in Wash Buffer as above. Clean precipitates  
25 (IB's) were recovered and were stored at -20 °C until use.

#### Refolding inclusion bodies

[0308] The IB's were weighed (144 mg) and dissolved in Genotech IBS buffer (1.44 ml). The resuspended IB's were incubated at 4 °C for one hour in an Eppendorf centrifuge tube. Insoluble material was removed by centrifugation at maximum speed in an Eppendorf  
30 centrifuge. Solubilized IB's were diluted to 4 ml final volume. Refolding of GST-ST3GalIII was tested in refolding buffer solutions containing cyclodextrin, polyethylene glycol (PEG), ND SB-201, or a GSH/GSSG redox system. One ml of solubilized IB's were diluted rapidly by pipetting into the refolding solution, vigorously mixed for 30-40 seconds, and then gently

**Table 3. GST-ST3GalIII activities after two separate folding experiments using GSH/GSSG system.**

GSH/GSSG	Conc	Activity
Refolding Trial 1	12x	182 U/L*
Refolding Trial 2	40x	531 U/L*

\*Activities reported here are Units per L refolded enzyme

#### Sialylation of glycoproteins using refolded GST-ST3 Gal III

[0311] Twenty  $\mu\text{L}$  of asialylated Transferrin ( $2\mu\text{g}/\mu\text{L}$ ) or asialylated Factor IX ( $2\mu\text{g}/\mu\text{L}$ ), was added to fifty  $\mu\text{L}$  of a buffer containing 50mM Tris, pH 8.0; and 150 mM NaCl, with 10  $\mu\text{L}$  of 100 mM  $\text{MnCl}_2$ ; 10  $\mu\text{L}$  of 200mM CMP-NAN; and 0.05% sodium azide. The reaction mixture was incubated with 30  $\mu\text{L}$  refolded GST-ST3GalIII at  $30^\circ\text{C}$  overnight or longer with shaking at 250 rpm. After the reactions were stopped, the sialylated proteins were separated on pH 7-3 IEF (Isoelectric focusing gel, Invitrogen) and stained with Comassie Blue according to manufacturer's guideline. Both Transferrin and Factor IX were sialylated by GST-ST3GalIII. (Data not shown.)

#### Refolding a rat liver ST3GalIII fused to an MBP tag.

[0312] Rat liver ST3GalIII was cloned into pMAL-c2x vector and expressed as a maltose binding protein (MBP) fusion, MBP-ST3GalIII, in inclusion bodies of *E.coli* TB1 cells. The refolded MBP-ST3GalIII was active and transferred sialic acid to LNnT, a sugar substrate, and to asialylated glycoproteins, for example asialo-transferrin.

#### Cloning ST3GalIII into pMAL-c2x vector

[0313] The rat liver ST3-GalIII nucleic acid was cloned into *Bam*HI and *Xba*I sites of the pMAL-c2x vector after PCR Amplification using the following primers:

Sense ST3BAMH1 5'-TAATGGATTCAAGCTACACTTACTCCAATGG<sup>(Seq ID No: 70)</sup>  
 Antisense: ST3XBA1 5'-GCGCTCTAGATCAGATACCACTGCTTAAGT<sup>(Seq ID No: 71)</sup>

[0314] Nucleotides encoding amino acids 28-374, e.g., the stem region and catalytic domain of ST3GalIII, were fused to the MBP amino acid tag.

[0315] Three other truncations of ST3GalIII were constructed and fused to MBP. The three ST3Gal III ( $\Delta 73$ ,  $\Delta 85$ ,  $\Delta 86$ ) inserts were isolated by PCR using the following 5' primers (ST3 BamHI  $\Delta 73$ ) TGTATCGGATCCCCTGGCCACCAAGTACGCTA<sup>(Seq ID No: 72)</sup>ACTT; (ST3 BamHI  $\Delta 85$ ) TGTATCGGATCCTGCAAACCCGGCTACGCTTCAGCCAT<sup>(Seq ID No: 73)</sup>; and (ST3

BamHI Δ86) TGTATCGGATCCAAACCCGGCTACGCTTCAGCCAT<sup>(SEQ ID No: 74)</sup> respectively, in pairs with the common 3' primer (ST3-Xho)<sup>(SEQ ID No: 75)</sup>

GGTCTCCTCGAGTCAGATACCACTGCTTAA<sup>(SEQ ID No: 75)</sup> Each PCR product was digested with BamHI and XhoI, subcloned into BamHI-XhoI digested pCWin2-MBP Kanr vector,

5 transformed into TB1 cells, and screened for the correct construct.

[0316] PCR reactions were carried out under the following conditions. One cycle at 95°C for 1 minute. One µl vent polymerase was added. Ten of the following cycles were performed: 94°C for 1 minute; 65°C for 1 minute; and 72°C for 1 minute. After a final ten minutes at 72°C, the reaction was cooled to 4°C.

10 [0317] All of the ST3GalIII truncations had activity after refolding. The experiments described below were performed using the MBP Δ73ST3GalIII truncation.

#### Expression of MBP-ST3GalIII in *E. coli* TB1 cells

[0318] The pMAL-ST3GalIII plasmid was transformed into chemically competent *E. coli* TB1 cells. Three isolated colonies containing TB1/pMAL-ST3GalIII construct were picked  
15 from the LB agar plates. The colonies were grown in five ml of LB media supplemented with 60 µg/ml carbenicillin at 37°C with shaking until the liquid cultures reached an OD<sub>620</sub> of 0.7. Two one ml aliquots were withdrawn from each culture and used to inoculate fresh media with or without 500 µM IPTG (final). The cultures were grown at 37°C for two hours. Bacterial cells were harvested by centrifugation. Total cell lysates were prepared heating the  
20 cell pellets in the presence of SDS and DTT. IPTG induced expression of MBP-ST3GalIII. (Data not shown.)

#### Expression of MBP-ST3GalIII and Purification of the inclusion bodies:

[0319] A one ml aliquot of TB1/pMAL-ST3GalIII overnight culture was inoculated into 0.5 liter of LB media with 50 µg/ml carbenicillin and grown to an OD<sub>620</sub> of 0.7. Expression  
25 of MBP-ST3GalIII was induced by addition of 0.5 mM IPTG, followed by overnight incubation at room temperature. The next day bacterial cells were harvested by centrifugation. Cell pellets were resuspended in a buffer containing 75 mM TrisHCl, pH 7.4; 100 mM NaCl; and 1 % glycerol. Bacterial cells were lyzed using a French Press. Soluble and insoluble fractions were separated by centrifugation for thirty minutes, 4°C, 10,000 rpm, Sorvall, SS 34 rotor). Soluble and insoluble fractions were separated by centrifugation for  
30 thirty minutes at 10,000RPM in a Sorvall, SS 34 rotor at 4°C.

buffer was supplemented with 0.3 mM Lauryl maltoside (LM); 0.1 mM oxidized glutathione (GSSG); 1 mM reduced glutathione (GSH) immediately before the addition of solubilized IB's. Two ml of solubilized IB's were added into 43 ml of refolding buffer in 50 ml sterile culture tube. The tube was placed on a rocker-shaker and gently shaken for 24 hours at 4°C.

- 5 The refolded protein was dialyzed in dialysis tubing (MWCO: 7 kD) against Dialysis Buffer (100 mM Tris HCl, pH 7.5; 100 mM NaCl; and 5 % glycerol) twice (in 10-20 volume excess buffer).

[0330] The large scale dialyzed, refolded MBP-Gal III was analyzed for ST3GalIII activity, and exhibited about 53.6 U/g IB.

#### 10 Example 2: Site Directed Mutagenesis of Human GnTI to Enhance Refolding

- [0331] A truncated human N-acetylglucosaminyltransferase I (103 amino terminal amino acids deleted) was expressed in *E.coli* as a maltose binding fusion protein (GnTI/MBP). The fusion protein was insoluble and was expressed in inclusion bodies. After solubilization and refolding, the GnTI/MBP fusion protein had low activity. The crystal structure of a truncated  
15 form of rabbit GnTI (105 amino terminal amino acids deleted) shows an unpaired cysteine residue (CYS123) near the active site. (See, *e.g.*, Unligil *et al.*, *EMBO J.* 19:5269-5280 (2000)). The corresponding unpaired cysteine in the human GnTI was identified as CYS121 and was replaced with a series of amino acids that are similar in size and chemical characteristics. The amino acids used include serine (Ser), threonine (Thr), alanine (Ala) and  
20 aspartic acid (Asp). In addition, a double mutant, ARG120ALA, CYS121HIS, was also made. The mutant GnTI/MBP fusion proteins were expressed in *E. coli*, refolded and assayed for GnTI activity towards glycoproteins.

[0332] Mutagenesis was done using a Quick Change Site-Directed Mutagenesis Kit from Stratagene. Additional restriction sites were introduced with some of the GnTI mutations.

- 25 For example an *Apa*I site (underlined, GGGCCCAC) was introduced into the GnTI ARG120ALA, CYS121HIS mutant, *i.e.*, CGC CTG → GCC CAC (changes in bold). The following mutagenic oligonucleotides were used to make the double mutant: GnTI R120A, C121H+, 5'CCGCAGCACTGTTCTGGGCCCACCTGGACAAGCTGCTG 3'<sup>(SEQ ID No: 76)</sup> and GnTI R120A, C121H- 5'CAGCAGCTTGTCCAGGTGGGCCCCGAACAGTGCTGCGG 3'<sup>(SEQ ID No: 77)</sup>  
30 (changes shown in bold). An *Asc*I site (underlined, GGCGCGCC) was introduced into the GnTI CYS121ALA mutant, *i.e.*, CTG → GCC (changes in bold). The following mutagenic oligonucleotides were used to make the GnTI CYS121ALA mutant: GnT1C123A+

5'AGCACTGTTTCGGCGCGCCCTGGACAAGCTGCTG 3' (SEQ ID No: 78)  
 5'CAGCAGCTTGTCCAGGGCGCGCCGAACAGTGCT 3' (SEQ ID No: 79).

[0333] The activity of the mutant proteins expressed in *E. coli* was compared to the activity of wild type GnT1 expressed in baculovirus. A CYS121SER GNT1 mutant was active in a TLC based assay. In contrast, a CYS121THR mutant had no detectable activity and a CYS121ASP mutant had low activity. A CYS121ALA mutant was very active, and a double mutant, ARG120ALA, CYS121HIS, based on the amino acid sequence of the *C. elegans* GnT1 protein (Gly14), also exhibited activity, including transfer of GlcNAc to glycoproteins. Amino acid and encoding nucleic acid sequences of the GnT1 mutants are provided in Figures 7-11.

[0334] A second GnT1 truncation was made and fused to MBP: MBP-GnT1(D35). Figure 35 provides a schematic of the MBP-GnT1 fusion proteins, and depicts the truncations, e.g., Δ103 or Δ35, and the Cys121Ser mutation (top). The bottom of the figure provides the full length human GnT1 protein. Mutations of Cys121 were also made in the MBP-GnT1(D35) protein.

[0335] Both fusion proteins were expressed in *E. coli* and both had activity for remodeling of the RNase B glycoprotein. Figure 36 provides an SDS-PAGE gel showing in the right panel the refolded MBP-GnT1 fusion proteins: MBP-GnT1(D35) C121A, MBP-GnT1(D103) R120A + C121H, and MBP-GnT1(D103) C121A. The left panel shows the activities for remodeling the RNase B glycoprotein of two different batches (A1 and A2) of refolded MBP-GnT1(D35) C121A at different time points. The MBP-GnT1 (D103) C121A also remodeled the RNase B glycoprotein. Data not shown.

#### Example 3: MPB fusions to GalT1.

[0336] The following fusions between truncated bovine GalT1 and MBP were constructed: MBP-GalT1 (D129) wt, (D70) wt or (D129 C342T). (For the full length bovine sequence, see, e.g., D'Agostaro *et al.*, *Eur. J. Biochem.* 183:211-217 (1989) and accession number CAA32695.) Each construct had activity after refolding. The amino acid sequence of the full length bovine GalT1 protein is provided in Figure 30. The mutants are depicted schematically in Figure 31 with a control protein GalT1(40) (S96A+C342T). See, e.g., Ramakrishnan *et al.*, *J. Biol. Chem.* 276:37666-37671 (2001).

[0337] MBP-GalT1 (D70) was expressed in *E. coli* strain JM109. After overnight induction with IPTG, inclusion bodies were isolated from the insoluble pellet after cells were

**Example 5: Refolding eukaryotic GalNAcT2.**

[0365] A truncated human GalNAcT2 enzyme was expressed in *E. coli* and used to determine optimal conditions for solubilization and refolding using the methods described above. The full length human GalNAcT2 nucleic acid and amino acid sequences are provided in Figures 13A and B. The sequences of the mutant protein, GalNAcT2(D51), are shown in Figures 14A and B. The mutant was expressed in *E. coli* as an MBP fusion protein, MBP-GalNAcT2(D51). Other GalNAcT2 mutants were made, expressed in *E. coli* and were able to be refolded: MBP-GalNAcT2(D40), MBP-GalNAcT2(D73), and MBP-GalNAcT2(D94). Data not shown. Details of the construction of the additional deletion mutants is found in USSN 60/576,530, filed June 3, 2004 and USSN 60/598,584, August 3, 2004, both of which are herein incorporated by reference for all purposes.

[0366] Cultures of bacteria expressing MBP-GalNAcT2(D51) were grown and harvested as described above. Inclusion bodies were purified from bacteria as described above.

Solubilization of the inclusion bodies was performed at pH 6.5 or at pH 8.0. After solubilization, MBP-GalNAcT2(D51) protein was refolded at either pH 6.5 or pH 8.0 using buffers A and B, *i.e.*, **Buffer A**: 55 mM MES pH 6.5, 550 mM Arginine, 0.055 % PEG3350, 264 mM NaCl, 11 mM KCl, supplemented with 1 mM GSH, 0.1 mM GSSG; and **Buffer B**: 55 mM TrisHCl pH 8, 550 mM Arginine, 0.055 % PEG3350, 264 mM NaCl, 11 mM KCl, supplemented with 1 mM GSH, 0.1 mM GSSG. After refolding, MBP-GalNAcT2(D51) protein was dialyzed and then concentrated. Figure 15 provides a demonstration of the protein concentration of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0.

[0367] A radiolabeled [<sup>3</sup>H]-UDP-GalNAc assay was performed to determine the activity of the *E. coli*-expressed refolded MBP-GalNAcT2(D51) by monitoring the addition of radiolabeled GalNAc to a peptide acceptor. The acceptor was a MuC-2 – like peptide having the sequence MVTPTPTPTC<sup>(SEQ ID NO: 80)</sup>. The peptide was dissolved in 1M Tris-HCl pH=8.0. See, *e.g.*, USSN 60/576,530 filed June 3, 2004; and US provisional patent application Attorney Docket Number 040853-01-5149-P1, filed August 3, 2004; both of which are herein incorporated by reference for all purposes. Figure 16 provides a demonstration of the enzymatic activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. Figure 17 provides a demonstration of the specific activity of refolded MBP-GalNAcT2(D51) after solubilization at pH 6.5 or pH 8.0 and refolding at pH 6.5 or pH 8.0. The highest activity levels were observed with MBP-GalNAcT2(D51) that

Human ST6GalNAc<sup>T</sup>  
 MRSC LWRCR HLSQGVQWSLLAVLVFFLFALPSFIKEPQTKPSRHQRTENIKERSLQS  
 LAKPKSQAPTRARRTTIYAEPVPENNALNTQTQPKAHTTGDRGKEANQAPPEEQDK  
 VPHTAQRAAWKSPEKEKTMVNTLSPRGQDAGMASGRTEAQSWKSQDTKTTQNGG  
 GQTRKLTASRTVSEKHQGAATTAKTLIPKSQHRMLAPTGA VSTRTRQKGVTTAVIP  
 PKEKKPQATPPPAPFQSPPTQRNQRLKAANFKSEPRWDFEEKYSFEIGGLQTTCPDSV  
 KIKASKSLWLQKLFPLNLTFLDSRHFNQSEWDRLEHFAPPFGFMELNYSLVQKVVT  
 RFPPVPQQQLLLASLPAGSLRCITCAVVGNGGILNNSHMGQEIDSHDYVFR LSGALIK  
 GYEQDVGTRTSFYGFTAFSLTQSLLILGNRGFKNVPLGKDVRYLHFLEGTRDYEWLE  
 ALLMNQTVMSKNLFWFRHRPQEA FREALHMDRYLLLHPDFLRYMKNRFLRSKTL D  
 GAHWRIYRPTTGALLLLTALQLCDQVSAYGFITEGHERFSDHYDYTSWKRLIFYINH  
 DFKLEREVWKRLHDEGIIRLYQRPGPGTAKAKN

**FIG. 38A**

Chicken ST6GalNAc<sup>T</sup>  
 MGFLIRRLPKDSRIFRWLLILTVFSFIITSFSALFGMEKSIFRQLKIYQSIAHMLQVDTQ  
 DQQGSNYSANGRISKVGLERDIAWLELNTAVSTPSGEGKEEQKTKVPVAKVEEAK  
 EKVTVPKPFPEVMGITNTTASTASVVERTKEKTTARPVPGVGEADGKRTTIALPSMKE  
 DKEKATVKPSFGMKVAHANSTSKDKPKAEPPASVKAIRPVTQAATVTEKKKLRAA  
 DFKTEPQWDFDDEYILDSSSPVSTCSESVRAKAAKSDWLRDLFLPNITLFDKSYFNV  
 SEWDRLEHFAPPYGFMEELNYSLVVEEVM SRLPPNPHQQLLLANSSSNVSTCISCAVVG  
 NGGILNNSGMGQEIDSHDYVFRVSGAVIKGYEKDVGTKTSFYGFTAYSLVSSLQNLG  
 HKGFKKIPQGKHIRYIHFLAVRDYEWL KALLLDKDIRKGFLNYYGRRPRERFDEDF  
 TMNKYLVAHPDFLRYLKNRFLKSKNLQKPYWRLYRPTTGALLLLTALHLCDRVSAY  
 GYTTEGHQKYS DHYDKWKRLV FYNHDFNLEKQVWKRLHDENIMKLYQRS

**FIG. 38B**

Mouse ST6GalNAc<sup>T</sup> protein beginning at residue 32 of the native mouse protein  
 DPRAKDSRCQFTWKNDASAQENQQAEPQVPIMTLSPRVHNKESTSVSSKDLKKQER  
 EAVQGEQAEGKEKRKLETIRPAPENPQSKAEPAAKTPVSEHLDKLPRTPGALSTRKTP  
 MATGAVPAKKKVVQATKSPASSPHPTTRRRQRLKASEFKSEPRWDFEEYSLDMSSL  
 QTNCASV KIKASKSPWLQNIFLPNITLFLDSGRFTQSEWNRLEHFAPPFGFMELNQSL  
 VQKVVTFRPPVRQQQLLLASLPTGYSKCITCAVVGNGGILNDSRVGREIDSHDYVFR  
 LSGAVIKGYEQDVGTRTSFYGFTAFSLTQSILILGRRGFQHVPLGKDVRYLHFLEGTR  
 NYEWLEAMFLNQTLAKTHLSWFRHRPQEA FRNALDLDRYLLLHPDFLRYMKNRFL  
 RSKTLDTAHWRIYRPTTGALLLLTALHLC DKVSAYGFITEGHQRFSDHYDYTSWKRL  
 IFYINHDFRLERMVWKRLHDEGIWLYQRPGQSDKAKN

**FIG. 38C**

	1		50
HSGALNAT1.pep	(1)MRKFAYCKVVLATSLIWWLLDMFLLLYFS-----	ECNKC	
HSGALNAT2.pep	(1)MRRRS--RMLLCFAFLWVLGIAYMYSGGGSALAGGAGGGAGRKEDWNEI		
Consensus	(1)MRK A KMLL A IWL F L	D N	
	51		100
HSGALNAT1.pep	(35)DEKKERGLPAGDVLEPVQKPHEGP-G-----EMGKPVVIPKEDQEKMKEM		
HSGALNAT2.pep	(49)DPIKKKDLHHSNGEEKAQSMETLPPGKVRWPDENQAYVGGIMVRSGQDP		
Consensus	(51)D K K L E Q P G D I D		
	101		150
HSGALNAT1.pep	(79)FKINQFNLMASEMIALNRSPLDVRLEGCKTKVYPDNLPTTSVVIVFHNEA		
HSGALNAT2.pep	(99)YARNKFNQVESDKLRMDRAIPDTRHDQCQRKQWRVDLPATSVVITFHNEA		
Consensus	(101)F N FN M SD I L RAIPD R D C K W LP TSVVI FHNEA		
	151		200
HSGALNAT1.pep	(129)WSTLLRTVHSHVINRSPRHMIEEIVLVDDASERDFLKRPLESYVKKLKVPV		
HSGALNAT2.pep	(149)RSALLRTVSVVLKKSPPHLIKEIILVDDYSN-----DPEDGALLGKIEKV		
Consensus	(151) S LLRTV SVI KSP HLI EIILVDD S P D L V		
	201		250
HSGALNAT1.pep	(179)HVIRMEQRSGLIRARLKGA AVSKGQVITFLDAHCEQIVGWLEPLLARIKH		
HSGALNAT2.pep	(194)RVLNRDRREGLMRSRVRGADAAQAKVLTFLDSHCEQNEHWLEPLLERRVAE		
Consensus	(201) VIR D R GLIRARLKGA A A VITFLDAHCEQ WLEPLL RI		
	251		300
HSGALNAT1.pep	(229)DRRTVVCPIIDVISDDTFEYMAGSDMTYGGFNWKLNFRWYPVFOREMDRR		
HSGALNAT2.pep	(244)DRTRVVSPIIDVINMDNFQYVGASADLKGGEFDWNLVFKWDYMTPEQRSR		
Consensus	(251)DR VV PIIDVI D F YMAAS GGF W L FKW M R		
	301		350
HSGALNAT1.pep	(279)KGDRTLFPVRTPTMAGGLESIDRDYFQEIGTYDAGMDIWGGENLEISFRIW		
HSGALNAT2.pep	(294)QGNPVAPIKTPMIAGGLEFVMDKFYFEELGKYDMMMDVWGGENLEISFRVW		
Consensus	(301) G PIKTP IAGGLE IDK YF EIG YD MDIWGGENLEISFRIW		
	351		400
HSGALNAT1.pep	(329)QCGGTLEIVTCSHVGHVFRKATPYTFPGGTGQIINKNNRRLAEVWMDEFK		
HSGALNAT2.pep	(344)QCGGSLEIIPCSRVGHVFRKQHPYTFPGGSGTVFARNIRRAAEVWMDEYK		
Consensus	(351)QCGGSLEII CS VGHVFRK PYTFPGGSG I KN RR AEVWMDEFK		
	401		450
HSGALNAT1.pep	(379)NFFYIISPGVTKVDYGDISSRVGLRHKLOCKPFSWYLENIYPDSQIPRHY		
HSGALNAT2.pep	(394)NFYYAAVPSARNVPYGNIQSRLELRKKLSCKPFWYLENVYPELRVPDHQ		
Consensus	(401)NFFY P V YG I SRL LR KL CKPF WYLENIYPD IP H		
	451		500
HSGALNAT1.pep	(429)FSLGEIRNVEITNQCLDNMARKENEKVGIFFNCHGMGGNQVFSYTANKEIRT		
HSGALNAT2.pep	(444)DIAFGALQOGTN-CLDTLGHFADGVVGVYECHNAGGNQEWALTKEKSVKH		
Consensus	(451) N TN CLD LA VGIF CH GGNQ FA T K IK		
	501		550
HSGALNAT1.pep	(479)DDLCLDVSKLN--GPVIMLKCHHLKGNQLWEYDPVKLTLOHVNSNQCLDK		
HSGALNAT2.pep	(493)MDLCLTVVDRAPGSLIKLQGCRENDSRQKWEQIEGNSKL RHVGSNLCLDS		
Consensus	(501) DLCL V I L C Q WE L HV SN CLD		
	551		584
HSGALNAT1.pep	(527)ATEEDSQVPSIRDCNGSRSQQWLLRNVTLP EIF-		
HSGALNAT2.pep	(543)RTAK-SGGLSVEVCGPALSQQWKFTLNLOQ----		
Consensus	(551) T S SI C A SQQW		

FIG. 44